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Articles

Mannitol-Specific Enzyme II of the Phosphoenolpyruvate-Dependent Phosphotransferase System of *Escherichia coli*: Physical Size of Enzyme II^{mtl} and Its Domains IIBA and IIC in the Active State†

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ABSTRACT: The size of enzyme II^{mtl} solubilized in the active state has been determined by size-exclusion chromatography under conditions that favor the association of the enzyme. The contribution of the detergent bound to the enzyme was determined by solubilizing the enzyme and running the TSK250 column in a number of detergents with decreasing micellar sizes. The size, expressed as the equivalent molecular mass of a globular protein, decreased from 315 kDa in decylPEG, to 275 kDa in octylPEG and octyl glucoside, and then to 245 kDa in cholate. Enzyme II^{mtl} is not active in the latter three detergents when at concentrations above their cmc values but still binds mannitol with high affinity without significant loss of sites. This, together with the full reversibility of the inactivation, is taken as evidence that the enzyme does not unfold or dissociate in these detergents. The sizes of the separated domains IIBA and IIC of enzyme II^{mtl} were 38 and 175 kDa, respectively. The cytoplasmic domain, IIBA, was monomeric at high concentration, whereas the membrane-bound domain, IIC, was associated at much lower concentration. Apparently, the sites that interact to keep enzyme II^{mtl} in the associated state are exclusively located in the membrane-bound domain.

Enzyme II^{mtl} of the bacterial P-enolpyruvate-dependent phosphotransferase system catalyzes the transport of mannitol across the cytoplasmic membrane in concert with the phosphorylation of mannitol. The phosphoryl group donor is a small cytoplasmic protein termed P-HPr [for a review, see Robillard and Lolkema (1988)]. The enzyme, a 68-kDa polypeptide, has been purified to homogeneity in the detergent decylPEG and shown to be fully active in the purified state (Jacobson et al., 1979; Roossien & Robillard, 1984a; Roossien et al., 1984; Elferink et al., 1990). The enzyme consists of three well-defined domains, termed IIA, IIB, and IIC. The latter is membrane-bound and constitutes about half of the protein. The other two domains, IIA and IIB, protrude into the cytoplasm and are hydrophilic (Grisafi et al., 1989; White & Jacobson, 1990; van Weeghel et al., 1991a,b).

Analysis of the mannitol phosphorylation kinetics catalyzed by enzyme II^{mtl} solubilized from the membrane by the detergent decylPEG indicated that an associated state of the enzyme is the catalytic unit (Lolkema & Robillard, 1990). The activity of the enzyme in the mannitol/mannitol-P exchange reaction

$$*\text{mannitol} + \text{mannitol-P} \rightarrow \text{mannitol} + *\text{mannitol-P} \quad (1)$$

a partial reaction of the overall reaction, increased quadratically with the enzyme concentration, indicative of the association of two particles. On the other hand, in the overall reaction

$$\text{mannitol} + \text{P-HPr} \rightarrow \text{mannitol-P} + \text{Hpr} \quad (2)$$

the specific activity increased 4-fold when measured over a large enzyme concentration range, showing that the dissociated state of the enzyme was capable of mannitol phosphorylation albeit with a lower specific activity than the associated state. It was concluded that the subunits of the enzyme II^{mtl} complex are, independently of one another, capable of catalyzing mannitol phosphorylation but that they interact cooperatively when in the associated state.

The monomeric and dimeric forms of enzyme II^{mtl} have been demonstrated by several techniques (Roossien & Robillard, 1984b; Roossien et al., 1986; Stephan & Jacobson, 1986; Pas et al., 1987; Khandekar & Jacobson, 1989). However, the enzyme is not active under the conditions under which these states of the enzyme were shown to exist. The functional studies indicated that the associated state of the enzyme is favored by high ionic strength (Lolkema & Robillard, 1990). In this paper, we will demonstrate the physical size of the associated state of enzyme II^{mtl} in the active state by size-exclusion column chromatography. The low ionic strength condition required for dissociation of the complex is not compatible with this technique. By running the membrane-bound domain IIC and the cytoplasmic domains IIBA separately, we will demonstrate that the sites of interaction that keep the enzyme in the associated state are exclusively located in the membrane-bound domain of the enzyme.

MATERIALS AND METHODS

Materials. [1-^{3}H(N)]-D-Mannitol (706.7 GBq/mmol) was purchased from NEN Research Products. Decylpoly(ethylene

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glycol) 300 (decylPEG) and octylpoly(ethylene glycol) 300 (octylPEG) were synthesized by B. Kwant in our laboratory. Octyl β-D-glucopyranoside (octyl glucoside) was obtained from Boehringer.

Membrane Preparation. *Escherichia coli* strain ML308-225 expressing wild-type enzyme II^{mtl} and strain ASL-1 with an enzyme II^{mtl} negative phenotype were grown as described in Lolkema and Robillard (1990). Strain ASL-1 was used as the host for plasmids pCIII and pNIII that code for domains IIBA and IIC, respectively (van Weeghel et al., 1991b). Cytoplasmic membranes from these cells were prepared essentialy as described by Lolkema and Robillard (1990) in a buffer containing 25 mM Tris, pH 7.5, 1 mM DTT, and 1 mM NaN_3.

Enzyme II^{mtl} was purified from membrane vesicles prepared from the ML308-225 strain essentially as described (Roossien & Robillard, 1984a; Robillard & Blaauw, 1987) with the following modifications. For reasons unknown, the enzyme II^{mtl} containing deoxycholate extract of the membranes did not bind any more to the hexylagarose resin under the high ionic strength conditions (250 mM NaCl) specified before. Binding could be reestablished by lowering the ionic strength of the extraction buffer. Therefore, the membranes were extracted with buffer containing 20 mM Tris, pH 8.4, 1 mM DTT, 1 mM NaN_3, 0.5% deoxycholate, and 25 mM NaCl. The extract was loaded on the hexylagarose column pre-equilibrated in the same buffer and washed with 1 column volume. The enzyme was eluted from the column by a gradient of 0–2% decylPEG in extraction buffer. The activity eluted at approximately 1% decylPEG.

The IIBA domain was purified as described (van Weeghel et al., 1991b).

Size-Exclusion Chromatography. The TSK-250 (60 × 0.75 cm) HPLC gel filtration column (Bio-Rad) was equilibrated in 50 mM Tris, pH 7.0, 50 mM KCl, 1 mM DTT, and a detergent at the indicated concentration. The output of the column was connected to a spectrophotometer equipped with a flow cell. The extinction at 215 nm was monitored continuously during a run, mainly to mark the start and end of the run. The column was loaded with 20 μL of sample and run at room temperature at a flow rate of 1 mL/min. Fractions of 0.5 mL were collected. The column was calibrated with the Bio-Rad calibration kit. The presence of different detergents had no significant effect on the elution positions of the proteins in this kit. Samples were prepared by solubilizing cytoplasmic membranes for 30 min at 4 °C in the column buffer with the appropriate detergent. Subsequently, undissolved material and larger aggregates were removed by spinning for 1 h at 100 000 rpm in a Beckman TL100 ultracentrifuge operated at 4 °C. The supernatant was immediately loaded on the column. The elution positions of the peak maxima were determined graphically, taking into account the asymmetric distribution of the data points over the peak. The error in the elution position was ±0.15 mL.

Activity Measurements. P-Enolpyruvate-dependent mannitol phosphorylation activity and mannitol/mannitol-P exchange activity were measured essentially as described (Lolkema & Robillard, 1990). Column fractions of runs in detergents other than decylPEG were diluted 100 times into the assay mixture containing 0.25% decylPEG to eliminate the inhibitory effect of these detergents. Runs with the IIBA domain were assayed by including 60 μg/mL membranes containing the IIC domain in the assay mixture. Runs with the IIC domain were assayed by including 8.8 μM purified domain IIBA in the assay mixture. Binding of [^{3}H]mannitol to enzyme II^{mtl} was measured by flow dialysis as described (Lolkema et al., 1990).

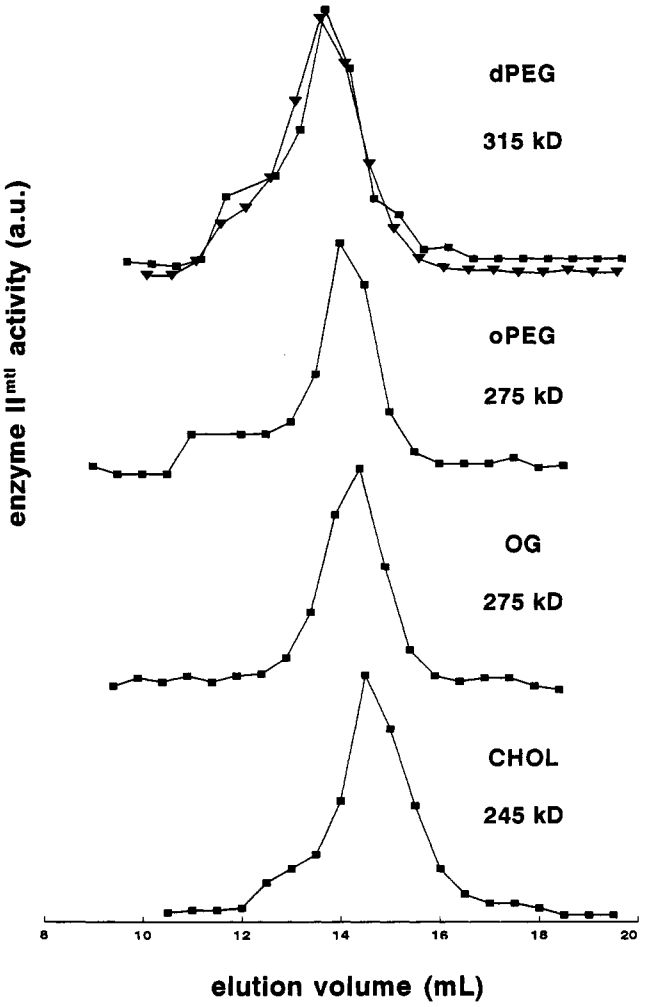

FIGURE 1: Elution profile of purified enzyme II^{mtl} (▼) and solubilized membranes (■) run on a TSK250 size-exclusion column. Cytoplasmic membranes (0.27 mg/mL) were solubilized, centrifuged, and run in 0.5% decylPEG (dPEG), 1% octylPEG (oPEG), 1.25% octyl glucoside (OG), or 4% cholate (CHOL). Purified enzyme II^{mtl} (24 nM) was run in 0.5% decylPEG. The recovery of activity was >75% in all runs.

RESULTS

Hydrodynamic Radius of the Associated State of Enzyme II^{mtl} Solubilized in DecylPEG. Cytoplasmic membranes derived from *Escherichia coli* cells that were induced for the mannitol transport system were solubilized in the detergent decylPEG and loaded on a TSK250 size-exclusion column. Enzyme II^{mtl} is fully active in the buffer used to run the column. The enzyme II^{mtl} activity eluted in a single peak at a position equivalent to the hydrodynamic radius of a (315 ± 15)-kDa globular protein (Figure 1, top track, ■). The experiment was repeated with purified enzyme II^{mtl} with identical results, indicating that other proteins in the membrane do not interfere with the physical state of enzyme II^{mtl} after solubilization (Figure 1, top track, ▼). The association/dissociation equilibrium of the enzyme II^{mtl} complex has been shown to depend strongly on the ionic strength of the buffer (Lolkema & Robillard, 1990). The high ionic strength of the column buffer, a requirement to minimize interaction between the macromolecules and the resin, favors the associated state of the enzyme. Nevertheless, the elution position of the enzyme will depend on the fraction of enzyme in the associated and

dissociated states. The degree of association of the enzyme was examined by loading purified enzyme II^{mtl} at concentrations of 1, 10, and 100 nM on the column. Mass action will favor the dissociated state as the concentration decreases. The experiments showed that the elution position was independent of the concentration of enzyme II^{mtl}, indicating that the enzyme is predominantly in the associated state in the concentration range used in the experiments. Therefore, the size of the associated state of enzyme II^{mtl} solubilized in decylPEG detergent is the equivalent of the size of a (315 ± 15)-kDa globular protein.

Contribution of the Detergent to the Hydrodynamic Radius. The size of enzyme II^{mtl} solubilized in detergent is determined both by the protein and by the detergent that is bound to the protein. At concentrations above the cmc of the detergent, the amount of detergent bound to the enzyme is on the order of the size of a micelle (Helenius et al., 1976). The micellar size of a detergent is inversely related to the cmc and decreases in the order decylPEG (≈35 kDa) > octylPEG (≈15 kDa) > octyl glucoside (≈8 kDa) > cholate (≈2 kDa). We have used this property of the different detergents to, more or less, titrate the contribution of the detergent out of the hydrodynamic radius of the enzyme II^{mtl} complex. Membranes were solubilized in octylPEG, octyl glucoside, and cholate at concentrations above their respective cmc's and loaded on the TSK250 column that was equilibrated in buffer in which decylPEG was replaced with these detergents (Figure 1). With decreasing micellar sizes of the detergents, the size of the enzyme II^{mtl} complex seemed to extrapolate to the equivalent of a (245 ± 12)-kDa globular protein. Assuming that the contribution of cholate to the size is negligible, the amount of decylPEG bound to enzyme II^{mtl} would be about 70 ± 27 kDa.

Activity of Enzyme II^{mtl} in Different Detergents. The activity of enzyme II^{mtl} in a number of detergents was determined by mixing cytoplasmic membranes with increasing concentrations of the detergents. The activity of enzyme II^{mtl} was assayed both in P-enolpyruvate-dependent mannitol phosphorylation and in mannitol/mannitol-P exchange (Figure 2, ● and ○, respectively). The former activity (reaction 2) requires, in addition to enzyme II^{mtl}, the general phosphotransferase components enzyme I and HPr, while for the latter activity (reaction 1) enzyme II^{mtl} is sufficient. Solubilizing cytoplasmic membranes in decylPEG results in an increase of the enzyme II^{mtl} activity in both reactions. It is believed that this is caused by breakdown of the vesicular structures which would make internally oriented mannitol binding sites on a significant fraction of the enzyme II^{mtl} population accessible to the substrate (Lolkema et al., 1992a). A similar increase of activity was seen with the detergent octylPEG but only in the exchange reaction. In the overall reaction, the activity drops rapidly to about 20% when the detergent concentration reaches the cmc of about 0.3%. The initial increase of the activity in the overall reaction in octyl glucoside is remarkable. A number of independent experiments showed that the phenomenon was reproducible. At octyl glucoside concentrations above the cmc of the detergent, the enzyme was inactive. The two ionic detergents cholate and deoxycholate inhibit the enzyme strongly when at concentrations above the cmc (0.4% and 0.08% under the experimental conditions, respectively). In both cases, the exchange reaction seems to be more sensitive to the detergent than the overall reaction.

To gain further insight into the state of enzyme II^{mtl} in these detergents, the binding of mannitol to the enzyme was analyzed. Enzyme II^{mtl} solubilized in decylPEG binds mannitol with an affinity constant in the submicromolar range (Lolkema et al., 1990; Pas et al., 1988). Table I shows that the enzyme solubilized in octylPEG, octyl glucoside, cholate, or deoxycholate has retained its high affinity for the substrate. Moreover, the number of binding sites did not change significantly. Though the enzyme is inactive in octyl glucoside, cholate, and deoxycholate, the mannitol binding domain IIC seems to be intact.

Size of the Membrane-Bound and Cytoplasmic Domains of Enzyme II^{mtl}. The base sequences coding for membrane-bound domain IIC and for cytoplasmic domains IIA plus IIB were subcloned from the MtlA gene, the structural gene for enzyme II^{mtl}, and expressed separately. Domain IIBA (IIA plus IIB) was purified and shown to be a soluble protein in the absence of detergent (van Weeghel et al., 1991b). Its molecular size was determined by loading domain IIBA at a concentration of 50 μM on the TSK250 column run in buffer with 0.5% decylPEG. The elution position was determined both by the extinction at 215 nm (not shown) and by the mannitol phosphorylation activity in the presence of membranes containing domain IIC (Figure 3, ▼). Both the extinction and the activity eluted at the position corresponding to a molecular mass of 38 ± 3 kDa. The molecular mass of domain IIBA from the primary sequence is 35 kDa. Membranes containing domain IIC were solubilized in buffer containing 0.5% decylPEG and loaded on the TSK250 column. The column fractions were assayed in a complementation assay with domain IIBA. The activity eluted at a position equivalent to the size of a (175 ± 9)-kDa globular protein. Assuming that the amounts of decylPEG bound to enzyme II^{mtl} and domain IIC are the same, the contribution of IIC to the size of the protein/detergent complex would be about the equivalent of a 105-kDa globular protein. The molecular mass of domain IIC from the primary sequence is 35 kDa.

DISCUSSION

The molecular weight of a membrane protein inferred from its elution position on a size-exclusion column calibrated with a set of globular proteins may differ from its true molecular weight because (i) a significant amount of detergent necessary to keep the enzyme in solution is bound to the enzyme and (ii) the shape of the enzyme/detergent complex is not globular. We have tried to eliminate the first problem by measuring the size of the enzyme II^{mtl}/detergent complex in a series of detergents with decreasing micellar sizes. The size of the complex seemed to extrapolate to the equivalent of a 245-kDa globular protein. Unfortunately, the enzyme was inactive in octyl glucoside and cholate, the two detergents that form the smallest micelles. Therefore, a change of association state or a complete or partial unfolding of the protein in these detergents cannot be excluded. Two experiments argue against the latter possibility: (i) the binding studies show that at least the binding domain is still intact, and (ii) the inactivation is fully reversible when the enzyme is diluted into a decylPEG-containing buffer. In addition, the binding assays provide evidence that the state of association does not change in these detergents. Binding studies of mannitol to purified enzyme II^{mtl} have revealed one high-affinity binding site per two molecules of enzyme (Pas et al., 1988). Dissociation of the enzyme will therefore lead to complete loss of binding when the binding site would be made at the interface of the two enzyme molecules or to a 2-fold increase in binding sites when the dissociated particles still bind mannitol with high affinity. Table I shows that neither of these two possibilities occurs. In conclusion, the

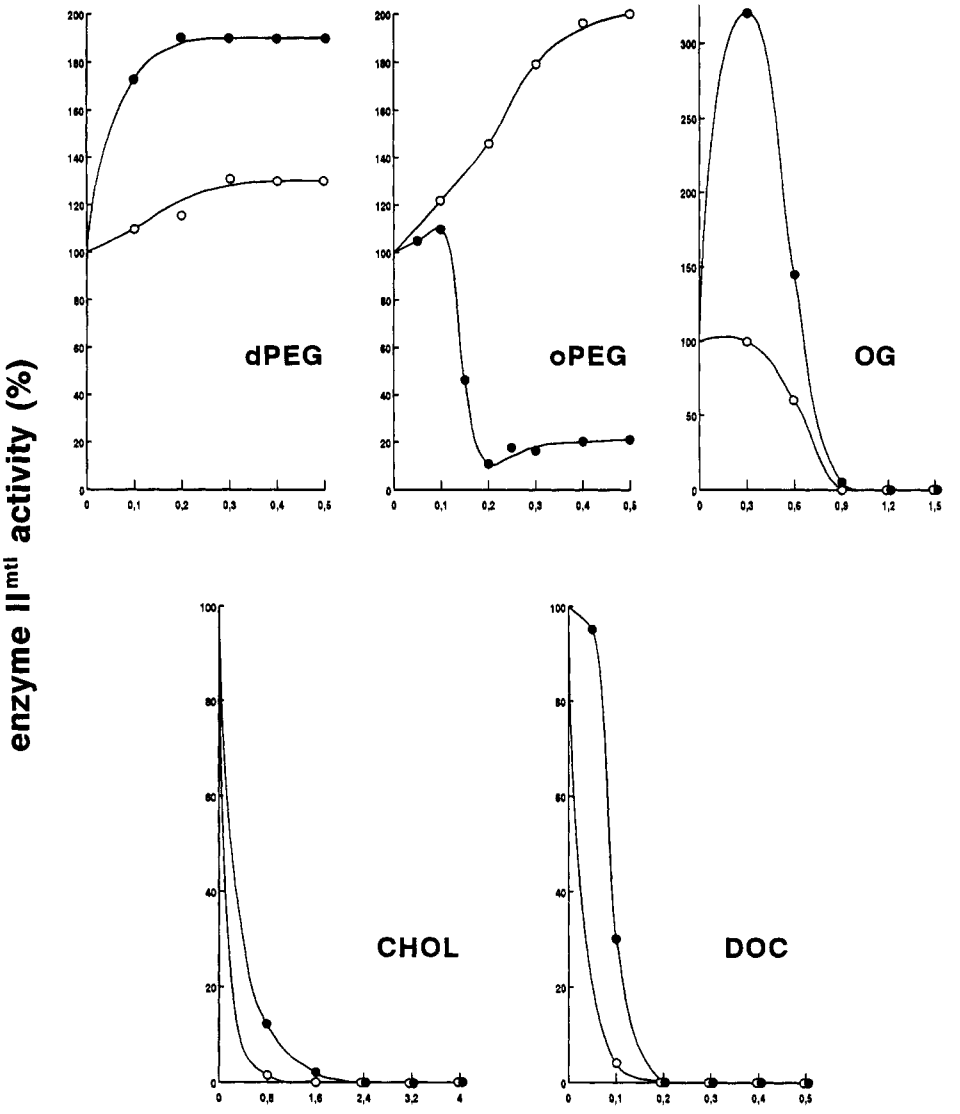

FIGURE 2: Activity of enzyme II^{mtl} in the overall (●) and exchange (○) reactions in decylPEG (dPEG), octylPEG (oPEG), octyl glucoside (OG), cholate (CHOL), and deoxycholate (DOC). Cytoplasmic membranes were mixed with the indicated concentration of detergent in a buffer containing 25 mM Tris, pH 7.5, 5 mM $MgCl_2$, and 5 mM DTT. The overall reaction. The membrane concentration was 1.1 μg/mL. The activity was measured in the presence of 5 mM P-enolpyruvate, 220 nM enzyme I, 3.6 μM HPr, and 20 μM [^{3}H]mannitol. The rate in the absence of detergent was 511 nmol/(min·mg). The exchange reaction. The membrane concentration was 2.2 μg/mL. The activity was measured in the presence of 2 mM mannitol-P and 0.5 μM [^{3}H]mannitol. The rate in the absence of detergent was 0.9 nmol/(min·mg).

Table I: Affinity (K_d^{mtl}) and Number of Sites (B_{max}) for the Binding of Mannitol to Enzyme II^{mtl} Containing Cytoplasmic Membranes Solubilized in Different Detergents

detergent	K_d^{mtl} (nM)	B_{max} (pmol/mg)
0.5% decylPEG	64	389
1.0% octylPEG	170	354
1.25% octyl glucoside	185	308
0.5% deoxycholate	25	380
4% cholate	42	119[a]

[a] A different membrane preparation.

data indicate that the contribution of the enzyme to the size of the active enzyme II^{mtl}/decylPEG complex of 315 kDa is about 245 kDa. This leaves about 70 kDa for the detergent which is twice the size of a decylPEG micelle.

A 245-kDa particle can accommodate three enzyme II^{mtl} molecules. Functional studies have shown that the associated state follows from the association of two particles; i.e., two monomers form a dimer, two dimers form a tetramer, etc. (Lolkema & Robillard, 1990). Therefore, the associated state must be a dimer (molecular mass = 136 kDa) with a shape that deviates significantly from a sphere. In this respect, it may be notable that the enzyme is half membrane protein and half cytoplasmic protein. The protein contribution to the size of the domain IIC/decylPEG complex of 105 kDa indicates that removal of the cytoplasmic domains from the enzyme II^{mtl} molecules results in a more spherical IIC dimer (molecular mass = 70 kDa).

Khandekar and Jacobson (1989) have determined the size of enzyme II^{mtl} by solubilizing cytoplasmic membranes in the detergent deoxycholate and subsequent loading on a Sephacryl S200 column run in a Tris buffer at pH 8.4. The elution profile of the enzyme II^{mtl} activity indicated a mixture of monomers and dimers with apparent molecular masses of 65 and 130 kDa, respectively. Our activity measurements indicate that enzyme II^{mtl} does not behave differently in deoxycholate

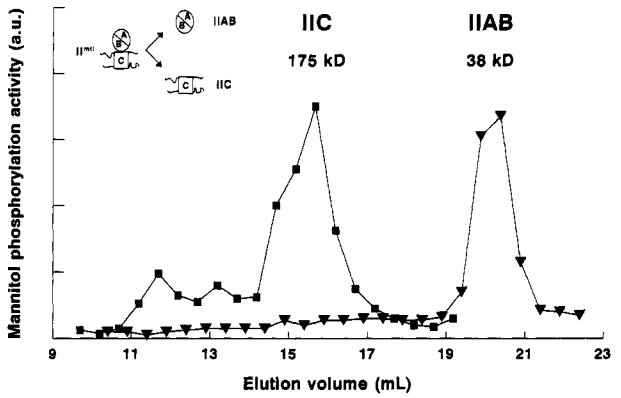

FIGURE 3: Elution profile of domain IIC (■) and domain IIBA (▼) on a TSK250 size-exclusion column run in 0.5% decylPEG. Cytoplasmic membranes containing domain IIC were treated at a concentration of 0.53 mg/mL as described in the legend of Figure 1 except that the centrifugation step was omitted. Domain IIBA was loaded on the column at a concentration of 50 μM. The recovery of activity from the column was 80% and 61% for IIC and IIBA, respectively.

than it does in the other detergents and, therefore, we tend to believe that the enzyme is in the associated state in deoxycholate at pH 7.5. Taken together, these data suggest that enzyme II^{mtl} would dissociate between pH 7.5 and pH 8.4. However, this is at variance with functional studies which indicate that association of the enzyme is favored at higher pH values (Lolkema & Robillard, 1990). We could not confirm the results of Khandekar and Jacobson because the TSK250 column should not be exposed to pH values higher than 7.

The runs with purified enzyme II^{mtl} showed that the enzyme was fully in the associated state at a concentration of 1 nM under the experimental conditions. The isolated membrane-bound domain IIC was in the associated state as well, and though we do not know the exact concentration that was loaded on the column, comparison with enzyme II^{mtl} concentrations from the same expression system indicated that the IIC concentration was well below 1 μM. On the other hand, the isolated cytoplasmic domains IIBA did not show any affinity for one another at a concentration of 50 μM. Apparently, the sites of interaction in the enzyme II^{mtl} dimer that keep the complex together are exclusively located in the membrane-bound domain. Kinetically, the membrane-bound domain catalyzes the transport of mannitol across the membrane. It is likely that the cooperativity between the two monomers in the dimer is at the level of the transport cycles.

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